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A hexamerin protein, AgSP-1, is associated with diapause in the boll weevil[1]

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Abstract

The objective of this research was to identify a reliable biochemical indicator for diapause (dormancy) in the boll weevil, *Anthonomus grandis*. Hemolymph polypeptides from reproductive and diapausing weevils were compared using denaturing sodium dodecyl sulpfate (SDS)-polyacrylamide gel electrophoresis (PAGE). A 77-kDa protein, which proved to be a hexamerin (AgSP-1), strongly correlated with morphological diapause characters in both male and female adult weevils. N-terminal sequence analysis identified the first 25 amino acids of the mature protein and was used to develop an antibody to AgSP-1. Anti-AgSP-1 reacted only with hemolymph from diapausing weevils of both sexes but not with hemolymph from reproductive weevils. Also, the yolk protein, vitellogenin (VG), inversely correlated with AgSP-1. When hemolymph VG was high, AgSP-1 was absent or barely perceptible.

Juvenile hormone regulates VG synthesis in most insect species. Juvenile hormone is reported to stimulate reproductive maturation in the boll weevil (Physiological Entomology 22 (1997) 261) and to be absent during diapause (Physiological Entomology 22 (1997a) 269). Therefore, the juvenile hormone (JH) mimic, methoprene, was used to assess the role of JH activity in the boll weevil for terminating diapause, stimulating reproductive maturation and possibly influencing AgSP-1 titers. Application of methoprene was not effective in activating reproductive development. Hemolymph from methoprene-treated, females contained VG and AgSP-1 titers that were similar to acetone-treated and untreated control weevils.

Using a genomic DNA library and 3′ RACE, two clones were isolated that yielded the complete sequence of AgSP-1 as well as a portion of the 5′ untranslated region. Northern blot analysis confirmed the presence of a 2.5 kB transcript for AgSP-1 in the fat body of diapausing weevils. AgSP-1 was also present in the fat body of reproductive weevils, but to a lesser extent. No sex-related differences in gene expression were observed; diapausing weevils of both sexes showed similar levels of AgSP-1 expression. An inverse correlation was observed between the VG transcript and AgSP-1 mRNA. VG was highly expressed in the fat body of reproductive females and only slightly expressed in tissue from diapausing females. Our data suggests that AgSP-1 is a diapause-specific protein in adult weevils and that JH, alone, is not effective in terminating diapause. © 2002 Published by Elsevier Science Ltd.

Keywords: Arylphorin; Storage protein; Boll weevil; *Anthonomus grandis*; Diapause

1. Introduction

Since its entry into the US, the boll weevil (*Anthonomus grandis* Boheman) has been the most serious economic pest of cotton in the production regions it infests. The rapid spread of this tropical insect across the US cotton belt was facilitated by its ability to survive temperate winters in a state of diapause. Brazzel and Newsom (1959) defined this dormant state as a reproductive diapause and described morphological changes in the reproductive structures of diapausing adult weevils, including cessation of gametogenesis and atrophy of the gonads. Following this initial report, substantial effort was directed towards elucidating the mechanisms controlling diapause induction in boll weevils.

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Photoperiod (Earle and Newsom, 1964; Lloyd et al., 1967; Sterling, 1972; Wagner and Villavaso, 1999), temperature (Earle and Newsom, 1964; Lloyd et al., 1967), adult or larval diet (Lloyd et al., 1967; Spurgeon and Raulston, 1996; Spurgeon and Raulston, 1998a; Spurgeon and Esquivel, 2000), and combinations of these factors (Cobb and Bass, 1968; Lloyd et al., 1967; Wagner et al., 1999) are implicated in the induction of diapause in the boll weevil. However, results and their interpretations have varied widely among the previous studies. Guerra et al. (1982) even questioned the use of the term 'diapause' to describe this dormancy in tropical and subtropical populations of weevils.

Several physiological characters have been investigated in an effort to better define boll weevil diapause. Increases in fat content and decreases in both water content and respiration rate are attributed to diapause (Brazzel and Newsom, 1959; Keeley et al., 1977; Lambremont, 1961; Lambremont et al., 1964). Although increases in lipids are generally associated with diapause, confounding influences of weevil age and diet (Betz and Lambremont, 1967; Lambremont et al., 1964), and geographical origin (Keeley et al., 1977) have been reported.

Results from studies of respiration rates are difficult to interpret. Although diapausing weevils generally exhibit lower respiration rates than their reproductive counterparts, variations in respiration rate have been associated with different laboratory cultures (Lambremont, 1961), geographical regions (Keeley et al., 1977), sex (Guerra et al., 1982), and adult age and diet (Guerra et al., 1983).

Studies of carbohydrates add little additional insight. Nettles et al. (1972) reported that trehalose levels in both reproductive and diapausing boll weevils remain fairly constant and do not vary significantly between the two physiological states. Nettles and Betz (1965) reported that glycogen levels increase in diapausing weevils when compared with reproductive weevils, but only when the weevils are fed cotton squares (flower buds). No such differences are observed when weevils are maintained on bolls. Betz and Lambremont (1967) indicated that glycogen levels are maximal in actively feeding 2-week-old adults and that glycogen content decreases in both diapausing and reproductive weevils when food is denied. Nettles et al. (1972) reported that when glycogen content is calculated on the basis of weevil dry weight, differences in glycogen levels relate to diet and sex without differences between reproductive and diapausing weevils.

Recently, JH production was implicated as an effective physiological character for defining diapause in the boll weevil. Taub-Montemayor and Rankin (1997) suggested that JH plays a regulatory role in reproductive development while absence of JH indicates diapause (Taub-Montemayor et al., 1997a). Elevated JH production is reported in reproductive, laboratory-strain females compared with field-collected females reared under short-day, low-temperature conditions. The field-collected females exhibit elevated juvenile hormone esterase (JHE) activity compared with reproductive, laboratory-strain weevils. However JHE activity in the latter group of weevils is not influenced by photoperiod or temperature conditions. In addition, males do not normally produce JH. Additional studies of field-collected weevils indicate a correlation between JHE titer and the ability to survive without food (Taub-Montemayor et al., 1997b). However, the relationship is variable among weevil cohorts and JHE titers vary widely among individuals within cohorts.

In the bean bug, *Riptortus clavatus*, and the Colorado potato beetle, *Leptinotarsa decemlineata*, the incidence of diapause correlates to the presence and abundance of a hemolymph polypeptide (De Kort and Koopmanschap, 1994; Chinzei et al., 1992; Lefevere et al., 1989). This hemolymph polypeptide belongs to a large family of proteins called the insect hexamerins and has been identified from a number of insects (for review see Burmester et al., 1998a; Haunerland, 1996; Kanost et al., 1990; Telfer and Kunkel, 1991). Likewise, the hemolymph polypeptide, VG, is the most abundant protein present during oocyte maturation (for review see Hagedorn and Kunkel, 1979; Raikhel and Dhadialla, 1992). Thus, expression patterns of hemolymph storage proteins and VG might serve as a basis for unambiguous biochemical discrimination of reproductive and diapausing boll weevils.

Successful examination of the boll weevil for presence of such a marker, however, will require reliable availability of both reproductive and diapausing individuals. Recent examinations of the influences of diet on boll weevil reproductive development (Spurgeon and Raulston, 1998b) and diapause induction (Spurgeon and Esquivel, 2000) have resulted in development of feeding regimes that appear to satisfy this need. Further, the morphological characters used to distinguish diapause in these studies strongly correlate with survival in the absence of food (D.W.S., unpublished data). Using a standard feeding regime (Spurgeon and Raulston, 1998b; Spurgeon and Esquivel, 2000) and defined morphological characters (Spurgeon and Raulston, 1998b), we identified a hemolymph polypeptide (AgSP-1) that strongly correlated with putative diapause in both male and female weevils. AgSP-1, an aromatic hexamerin, was abundant in diapausing weevils and was absent or barely detectable in reproductive weevils. VG was inversely correlated with AgSP-1 in females at the level of transcription and translation. Here, we report the molecular characterization of AgSP-1 and its synthesis and expression during diapause in the boll weevil.

2. Materials and methods

2.1. Experimental animals

Boll weevil adults of known age were reared from oviposition-punctured flower buds (squares) collected from commercial cotton fields. Infested squares were held in a screened plexi-glass cage at 29.4 ± 1°C under a 13:11 h (L:D) photoperiod. Beginning five days after collection, squares were carefully opened and pupae were removed. Squares containing larvae were returned to the cage and resulting pupae were harvested on subsequent days. Pupae were held in groups of 35–50 in petri plates ($100 \times 15 mm^2$) containing a thin layer of moistened vermiculite and under the same environmental conditions as infested squares. Newly eclosed adults were removed from the pupal plates twice daily and sexed using the technique of Sappington and Spurgeon (2000).

2.2. Reproduction- and diapause-inducing feeding regimes

Reproductive and diapausing weevils were obtained by manipulating adult diet as described by Spurgeon and Raulston (1998a). Reproductive weevils were obtained by confining newly eclosed adults individually in petri plates ($100 \times 15 mm^2$). A single, fresh square with intact bracteoles and a bud diameter of 6–9 mm was supplied to each weevil daily. Drinking water was supplied by a short (~1 cm) section of cotton dental wick saturated with deionized water. Diapausing weevils were obtained by confining mixed-sex groups of 25 newly-eclosed weevils (12 ♂, 13 ♀) in 473-ml cardboard cartons with screened lids. These groups were fed 6–9 mm diameter squares with bracteoles removed at a rate of one square per five weevils daily. Water was provided in a 29.5-ml plastic cup with a section of cotton dental wick extending through the lid.

Weevils at 9- or 12-day-old, were punctured near the midline of the prothorax with the tip of a 20-gauge hypodermic needle and hemolymph was collected in a 1.0-μl micro-capillary tube (Drummond Scientific Co., Broomall, PA) as it exuded from the puncture. Bled weevils were then dissected to determine physiological status and to collect fat body samples.

Condition of the fat body, testes, and ovaries were assessed by dissection. After removal of the elytra and wings, the dorsal abdominal cuticle was peeled away. Exposed fat bodies of both sexes were classified as lean, intermediate, or fat. Fat bodies classed as lean varied considerably in abundance, and were sheet-like and translucent to opaque, but not bright white in color. Fat bodies classed as intermediate were bright white in color, composed of well-formed globules, and obscured substantial portions of other internal organs. Fat bodies classed as fat were similar in color and composition to those classed as intermediate, but completely or nearly completely obscured the other internal organs. Fat bodies rated as fat or intermediate were considered hypertrophied and characteristic of diapausing weevils. Ovaries were classified as undeveloped, containing pre-vitellogenic oocytes, containing oocytes with visible yolk, or containing chorionated eggs. Testes were classified as reproductive or atrophied according to the descriptions by Spurgeon and Raulston (1998b). Reproductive testes were translucent and developing sperm were visible in the testis lobes. Atrophied testes were opaque with whitish to bright yellow external fat deposits and were normally much smaller than reproductive testes. Females with hypertrophied fat bodies and with ovaries that were undeveloped or that contained only pre-vitellogenic oocytes were considered to be in a reproductive diapause. Females with ovaries containing oocytes with visible yolk or chorionated eggs were considered reproductive regardless of fat body condition. Likewise, males with hypertrophied fat bodies and atrophied testes were considered to be in diapause while males with reproductive testes were considered reproductive regardless of fat body condition.

2.3. Response of diapausing female weevils to methoprene

2.3.1. Preparation of diapausing females

To obtain the highest possible incidence of diapause, experimental weevils were fed a diet of cotton fruit (bolls) as described by Spurgeon and Esquivel (2000), and reproductive weevils were further eliminated by starvation during a food-free period between diapause induction and treatment applications. After newly-eclosed females were sufficiently sclerotized to walk they were transferred to screened $20 \times 20 \times 20 cm^3$ plexi-glass cages and held at 29.4 ± 1°C under a 11:13 h (L:D) photoperiod. Adults eclosing over a period of five consecutive days, of which 90% eclosed in a 3-day period, were pooled until the desired number of weevils were obtained. Weevils were supplied a diet of one boll (20–25 mm diameter) per 10 weevils three times weekly, and drinking water as previously described.

When the median age was 14 days, weevils were transferred to an identical cage containing only a water supply and a refuge of crumpled craft paper ($30 \times 45 cm^2$). These weevils were held without access to food for 30 days in an environmental chamber at 18.3 ± 1°C under a 11:13 h (L:D) photoperiod. At the end of the starvation period, approximately 20 weevils were randomly assigned to either the methoprene treatment or one of two control groups.

2.4. Administration of methoprene

Previous studies indicated that the JH mimic, methoprene, was effective in stimulating VG synthesis in *A. grandis* (Taub-Montemayor and Rankin, 1997; Taub-Montemayor et al., 1997b). Therefore, a methoprene dose of 1 μg per treatment was selected based on an initial-dose response study. A single dose of methoprene ranging from 0.1 to 10 μg was administered to newly-eclosed adult females maintained under the diapause-inducing feeding regime for seven days. At day 7, hemolymph samples were collected and analyzed. Females treated with methoprene concentrations of 1–10 μg showed VG presence with comparable frequency, however, there was variability for these responses among individual animals in all the treatment groups (data not shown). The variability in the dose response may have resulted from the short time period used to induce diapause. Thus, to ensure diapause, boll weevils were fed the diapause-inducing diet for 14 days and then starved for an additional 30 days as described before. The lowest dose (1 μg) was selected to minimize possible pharmacological effects. This dose was applied a total of three times at three-day intervals in an attempt to produce a sustained level of JH activity in vivo, similar to what might occur naturally.

At the end of the 30 day starvation period, methoprene-treated and control groups were transferred to respective cages identical to the survival cage and held in the environmental chamber for the duration of the experiment. Weevils of the methoprene group were treated topically three times with 1 μg methoprene in 1 μl acetone applied to the ventral surface of the abdomen on days 0, 3, and 6 following the starvation period. Weevils of one control group were similarly treated with 1 μl acetone. The experiment was replicated twice. The second replication included an additional control group of approximately 20 untreated weevils. Hemolymph samples were collected and weevils were dissected nine days after initial treatments were applied. Fat body and ovary conditions were assessed as previously described. Hemolymph samples were analyzed for the presence of VG and AgSP-1 polypeptides by sodium dodecyl sulpfate (SDS)-polyacrylamide gel electrophoresis(PAGE).

2.5. Hemolymph sample preparation

Hemolymph samples for SDS-PAGE analysis were diluted in ice-cold Tris-buffered saline homogenization buffer (=TBS-HB) (20 mM Tris; 0.15 M NaCl; 5 mM EDTA, pH=7.5) (Martinez and Wheeler, 1991) containing a Complete™ Mini protease inhibitor cocktail tablet (Boehringer Mannheim, Indianapolis, IN) (Lewis et al., 2001). Hemolymph protein concentrations were determined spectrophotometrically using the Micro BCA Assay Reagent Kit (Pierce, Rockford, IL). Following protein quantification, the hemolymph samples were separated by SDS-PAGE.

2.6. SDS-PAGE

Hemolymph samples were brought to a final protein concentration of 8 μg and diluted in an equal volume of 2× sample buffer as described by Lewis et al. (2001) (5% (w/v) SDS; 0.126 M Tris–Cl, pH=6.8, 12.5 % (w/v) glycerol, 0.004% bromophenol blue, 2% (w/v) NaCl; 10% (v/v) β-mercaptoethanol). Each lane received hemolymph collected from a single animal. SDS-PAGE was performed according to Laemmli (1970) and modified to use polyacrylamide gels consisting of exponential gradients of 6–16% acrylamide and 0.16–0.85% bis-acrylamide (Sowa et al., 1991). A 4.5% stacking gel was used with all exponential gradient-polyacrylamide gels and molecular weights were estimated using the Mark12™ (Invitrogen, Carlsbad, CA) unstained standard (15 μl/lane). Electrophoresis was carried out at 5 W constant power until the tracking dye (bromophenol blue) migrated off the gel. The protein bands were stained using Gel Code® Blue Stain Reagent (Pierce) and destained with distilled water.

2.7. N-terminal sequencing

SDS-PAGE analysis indicated that a 77-kDa hemolymph polypeptide was the dominant component in weevils displaying diapause characters (Fig. 1). To deter-

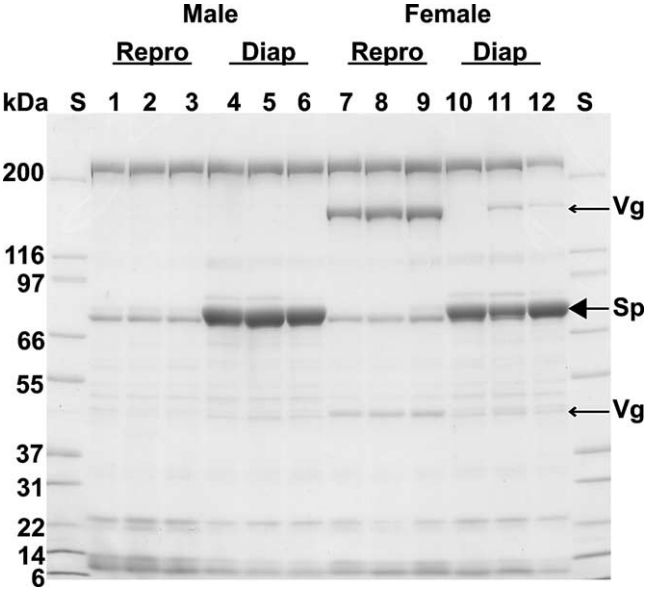

Fig. 1. Coomassie blue-stained SDS-PAGE (exponential gradient consisting of 6–16% acrylamide and 0.16–0.85% bis-acrylamide) gel comparing hemolymph polypeptides from reproductive and diapausing *A. grandis*. Each lane was loaded with 8 μg of soluble polypeptides from the hemolymph of a single, reproductive male (lanes 1–3), diapausing male (lanes 4–6), reproductive female (lanes 7–9) and diapausing female (lanes 10–12). AgSP-1 (arrow), both subunits of VG (arrow), and the molecular weight standards (S) are indicated.

mine the identity of this polypeptide, hemolymph from four weevils showing diapause characters were diluted in TBS-HB:2× sample buffer (1:1) and separated on a 7.5% SDS polyacrylamide gel (Laemmli, 1970) containing a 4.5% stack. To remove unpolymerized acrylamide, 1 mM sodium thioglycolate was added to the upper buffer and the gel was allowed to pre-electrophorese for 30 min prior to loading. The samples were denatured at 65 °C for 15 min. The gel was electrophoresed at 20 mA through the stack after which the amperage was increased to 25 mA. Electrophoresis ended once the dye front reached the bottom of the gel. Following SDS-PAGE separation, the hemolymph polypeptides were transferred to a polyvinylidene fluoride (PVDF) membrane (BioRad, Hercules, CA) in cyclohexylamino-propanesulfonic acid (CAPS) buffer (10 mM CAPS, 10% methanol, pH 11.0) for 1 h at 200 V. The membrane was stained with Coomassie blue for 5 min (0.1% Coomassie blue R-250, 50% methanol, 1% acetic acid) and then rinsed several times with distilled water to visualize the bands. N-terminal analysis of the 77-kDa polypeptide was performed by the Protein Chemistry Laboratory (Texas A & M University, College Station, TX).

2.8. Antibody production

Anti-AgSP-1 (Alpha Diagnostic International Inc., standard custom antiserum service, San Antonio, TX) polyclonal antibodies were raised in a rabbit against a synthetic peptide based on the N-terminal sequence combined with purified hemolymph AgSP-1 that had been collected from diapausing weevils. Purified hemolymph AgSP-1 was obtained by separation with SDS-PAGE and micro-elution using a Gel-Eluter (Hoefer, San Francisco, CA). Initial results using Anti-AgSP-1 were unsatisfactory, thus the antibody was affinity purified against the synthetic peptide alone (Research Genetics, Huntsville, AL).

2.9. Immunoblotting

Hemolymph from reproductive and diapausing weevils of both sexes was collected and its proteins were separated in an 8–16% exponential SDS-polyacrylamide mini-gel (BioRad, Hercules, CA). After determination of protein concentration, each sample containing 8 μg total protein was loaded in duplicate lanes along with a BioRad prestained SDS-PAGE standard (10 μl/lane). Electrophoresis was carried out at 200 V for 3 h. One half of the gel was Coomassie blue stained and the other half was transferred onto nitrocellulose using the Trans-Blot SD Semi-Dry Transfer System (BioRad) for 30 min at 25 V. The membrane was exposed to the affinity-purified anti-AgSP-1 (1:100) and antigen/antibody interaction was detected using goat anti-rabbit IgG (whole molecule)-Peroxidase secondary antibody (Sigma, St. Louis, MO) according to the manufacturer's recommendations. Chemi Glow™ West chemiluminescent substrate (Alpha Innotech Corporation, San Leandro, CA) was used to react with the immobilized peroxidase, and the reaction was visualized and documented using a computer-interfaced Chemi-Doc system from BioRad.

2.10. Preparation of DNA probes

2.10.1. Ag storage protein probe

A 1.0-kB DNA fragment was developed for genomic DNA library screening and Northern blot analysis using the RNA PCR Core Kit (Perkin Elmer, Wellesley, MA). Manufacturer's instructions were followed for reverse transcription (RT) of total RNA from the fat body of *A. grandis* diapausing males. The reverse primer used for RT was oligo-dT_{16} which was supplied in the kit. Degenerate primers (forward: GAY CAY GCI TTY YTI GAR AA; reverse: TTC GAA GTC CTT AGC RTA GTA) were used for amplification of the cDNA by polymerase chain reaction (PCR). Primers were designed using the sequence obtained from the N-terminal analysis of AgSP-1 and from published storage protein sequences (Fujii et al., 1989; Jamroz et al., 1996; Koopmanschap et al., 1995; Sakurai et al., 1988a). The PCR reaction mix consisted of 1× PCR buffer, 2 mM $MgCl_2$, 0.20 mM dNTP-mix, 0.05 U AmpliTaq® (Perkin Elmer). The PCR cycles were 94 °C/2 min; 35 cycles of 94 °C/30 s, 45 °C/1 min, 72 °C/2 min; 72 °C/7 min. The primary PCR reaction (2 μl) was reamplified for an additional 25 cycles under the same conditions. The resulting band was cleaned from agarose using the Qiaex II extraction kit (Qiagen, Valencia, CA) and cloned into pGEM®-T easy vector (Promega, Madison, WI). The fragment was sequenced by the DNA Technologies Laboratory (Texas A & M University, College Station, TX) using ABI PRISM Big Dye Terminator Cycle Sequencing Core Kit with Amplitaq® DNA polymerase (Perkin Elmer).

2.10.2. Ag VG probe

A 0.5-kB DNA fragment (AgVG) was developed as a probe for Northern blot analysis using total RNA from the fat body of female reproductive *A. grandis*. The RNA PCR core kit (Perkin Elmer) was used as described before. VG reverse primer (see later) was used for the RT reaction as well as amplification of the target cDNA. The primers (Vg forward: TAT CAA CAG CGC TTT ATT GTC ATG C; Vg reverse: ACA ATG GCG CAA CTC TCC TTC) were designed based on the published *A. grandis* VG gene sequence (location of sequence=+6010–6538) (Trewitt et al., 1992). The same PCR reaction mix was used as described for the 1.0-kB AgSP-1 storage protein probe. The PCR cycles were 94 °C/2 min; 35 cycles of 94 °C/30 s, 60 °C/1 min, 72 °C/2 min; 72 °C/7 min. The primary PCR reaction (2 μl) was

reamplified for an additional 25 cycles with the same PCR conditions. The resulting band was cleaned, cloned, and sequenced as described for the *A. grandis* storage protein probe.

2.11. Genomic library analysis

The boll weevil genomic DNA library was constructed in λgt11 by DNA Technologies, Inc. (Gaithersburg, MD) and primarily was used to obtain the 5′ untranslated region of the putative storage protein gene. Plaques from the λgt11 genomic library were propagated on *E. coli* strain Y1088 according to Sambrook et al. (1989). Approximately 7.5×10^5 plaques were screened at high stringency (0.6 Na^+, 65 °C hybridization; 15 mM Na^+, 65 °C wash) using the 1.0-kB ^{32}P-labeled storage protein probe, and a single 2.2-kB clone was obtained. The resulting clone was digested with *Eco*RI, ligated into pBluescript and sequenced.

2.12. The 3′ rapid amplification of cDNA ends (RACE)

To obtain the full-length cDNA gene sequence of the *A. grandis* putative storage protein, 3′ RACE was performed using the 3′ RACE System (Gibco BRL®, Carlsbad, CA). Manufacturer's instructions were followed for reverse transcription of total RNA from the fat body of *A. grandis* diapausing males. A gene specific forward primer (GSP1: ACC TTT TGG GCT ACT CTT ACA CTC C) along with a kit-supplied reverse primer was used for amplification using conditions specified in the kit. The PCR cycles were 94 °C/2 min.; 35 cycles of 94 °C/30 s, 55 °C/1 min, 72 °C/2 min; 72 °C/7 min.

2.13. Computer-assisted analysis

Translation of the nucleotide sequence was accomplished with the assistance of Molecular Biology Shortcuts (MBS) translator through the MBS-e-mail server. The National Center for Biotechnology Information BLAST e-mail server was used to compare the sequence results with other genes available through the GenBank database (Altschul et al., 1990). Protean (DNASTAR, version 1.17) was used to deduce the amino acid composition and MegAlign (DNASTAR, version 1.05, Madison, WI) was used for the initial multiple amino acid sequence alignment which was adjusted by eye, when necessary. Maximum parsimony and neighbor-joining bootstrap analyses were perfomed using PAUP 4.0 (Swofford, 1998).

2.14. Northern blot analysis

Northern blot analysis was used to determine the size and relative abundance of the AgSP-1 transcript from the fat body of reproductive and diapausing *A. grandis* weevils. SDS-PAGE analysis indicated an inverse correlation between VG and AgSP-1 protein in females, therefore, VG gene expression was also examined.

Total RNA (10 μg for the VG blot; 5 μg for the SP blot) was extracted from the fat body (Chomczynski and Sacchi, 1987; Jamroz et al., 1996; Puissant and Houdebine, 1990) of reproductive females, reproductive males, diapausing females and diapausing males. The samples were separated in a 1.5% agarose/formaldehyde gel (0.7 M formadlehyde, 1× MOPS) and transferred to a positively charged nylon filter (Hybond XL, Amersham Pharmacia, Piscataway, NJ) with 20× SSC as transfer medium. Two blots were used for the analyses; both blots consisted of RNA from the same extracted preparations. The blots were hybridized with either the 0.5-kB ^{32}P-labeled AgVG probe or the 1.0-kB ^{32}P-labeled storage protein probe. Hybridization was performed at 48 °C (UltraHyb, Ambion, Austin, TX) for 12–14 h. Washes were performed at 65°C (2*5min—2 × SSC, 0.1% SDS; 2*15min—0.1 × SSC;0.1%SDS). Transcripts were visualized by autoradiography (exposuretime = ~ 1h).

3. Results

3.1. Comparison of hemolymph polypeptides from reproductive and diapausing A. grandis

Hemolymph polypeptides were analyzed by SDS-PAGE for male and female weevils showing either reproductive or diapause morphological characters. A band that migrated at approximately 77-kDa was the major polypeptide in diapausing weevils (Fig. 1). The 77-kDa band was abundant in both male and female diapausing weevils and was reduced or absent in reproductive weevils.

In females, a large polypeptide with a molecular weight of approximately 160-kDa inversely correlated with the 77-kDa band (Fig. 1). The 160-kDa polypeptide was the dominant polypeptide in reproductive females and was absent or barely perceptible in diapausing females. Trewitt et al. (1992) characterized the VG gene in the boll weevil and reported that the translated product was composed of two subunits with molecular weights of 160 and 47 kDa, based on migration in SDS-PAGE analyses. Thus, the large polypeptide we observed in the hemolymph of reproductive females was likely VG. A 47-kDa polypeptide coincided with the presence of the 160-kDa band and is likely the smaller subunit.

3.2. N-terminal sequencing and immunoblotting

The 77-kDa polypeptide was isolated and sequenced by the Protein Chemistry Laboratory to determine its

identity (Texas A & M University, College Station, TX). N-terminal analysis yielded a 25-amino acid sequence (SPVGDGKHYKTVDHAFLEKQQKVLS) that showed high similarity with sequences of other insect hexamerin proteins present in the GenBank database. Given its homology to storage proteins, the 77-kDa polypeptide was named AgSP-1. This initial sequence was used to prepare the synthetic peptide for raising anti-AgSP-1 polyclonal antibodies, and was also used to design the degenerate forward primer for RT-PCR of the 1.0-kB *A. grandis* storage protein probe.

Immunoblotting of hemolymph polypeptides from diapausing and reproductive weevils with anti-AgSP-1 showed that AgSP-1 is a diapause-specific biochemical marker (Fig. 2). Anti-AgSP-1 reacted with male and female hemolymph polypeptides from diapausing weevils but did not react with hemolymph polypeptides from reproductive weevils of either sex.

3.3. SDS-PAGE analysis of methoprene-treated diapause-induced female A. grandis

The inverse correlation we observed between VG and AgSP-1 led us to evaluate a potential role for JH in boll weevil diapause. Results of methoprene treatment on hemolymph VG and AgSP-1 in diapause termination are shown in Fig. 3. Typical results are shown for 10–12 females from each treatment. There were no significant differences in any of the treatment regimes or in either replication. VG was absent or barely visible in methoprene-treated females indicating that the methoprene treatment failed to stimulate VG synthesis consistently. In the no-treatment group, two of the females showed reproductive characters in that VG was the prominent polypeptide and AgSP-1 was absent (lanes 3 and 10). Also, the fat bodies of these two animals were rated lean (as opposed to fat or intermediate which is typical of diapause) and the ovaries contained oocytes with visible yolk and chorionated eggs. The same was also observed in two of the acetone-treated females (lanes 5 and 9). Given that the reproductive characters occurred in the control groups and not in the methoprene-treated group, the results suggest that this regimen of methoprene had no effect on VG synthesis.

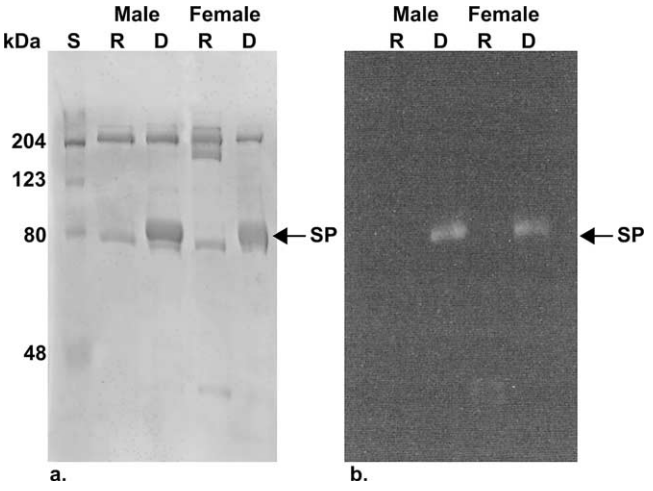

Fig. 2. Western blot analysis of AgSP-1 in *A. grandis*. Hemolymph from reproductive (R) and diapausing (D) weevils of both sexes were separated on an 8–16% exponential SDS-polyacrylamide gel. One half of the gel was (a) Coomassie blue stained; and (b) the other half was transferred onto nitrocellulose and probed with polyclonal antibodies to the N-terminus of AgSP-1. Each lane was load with hemolymph samples containing 8 μg of protein from a single animal. Each sample was loaded in duplicate. AgSP-1 (SP) and the molecular weight standards (S) are indicated.

3.4. Genomic DNA library screening and 3′ RACE analysis

The *A. grandis* genomic DNA library was screened with a ^{32}P-labeled storage protein probe to obtain a single 2.2-kB clone. 3′ RACE was used to generate an overlapping 1.0-kB fragment. Thus, the combined sequence from both the 2.2-kB genomic DNA clone and the 1.0-kB 3′ RACE clone yielded the complete DNA sequence of AgSP-1 (Fig. 4).

The genomic DNA clone represented 495 bp of the 5′ untranslated region and 1426 bp of the coding region (Fig. 4). Five small introns (54–60 bp) were observed (data not shown). The placement of the introns was verified by PCR amplification using gene specific primers ($^{+}$1–$^{+}$481, $^{+}$1080–$^{+}$1400) as well as comparing the sequence of the genomic clone with the sequence of the 1.0-kB storage protein probe. The transcription start site was deduced from direct sequencing of the *A. grandis* 2.2-kB genomic clone and was located 26 bp upstream of the ATG translation start codon. The transcription start site in AgSP-1 (ATCAGTT) is homologous with the consensus sequence observed in other insect genes (Hultmark et al., 1986). The TATA box is located 32 bp upstream of the transcription start site (Breathnach and Chambon, 1981; Gannon et al., 1979).

The open reading frame of AgSP-1 was composed of 2202 nucleotides encoding a 734 amino acid protein. The first 17 amino acids of the deduced sequence is a putative signal peptide common to many secretory proteins. Thus, the mature protein consists of 717 residues with an expected Mr of 85.3 kDa. The first 25 residues of the mature protein matched the 25-amino acid N-terminal sequence (Fig. 4). The deduced amino acid composition of AgSP-1 reveals that phenyalanine (Phe) and tyrosine (Tyr) comprise ~21% (mol %) of the total amino acid content (Table 1). Three potential N-linked glycosylation sites were identified (Fig. 4).

The 3′ untranslated region is present in the 1.0-kB 3′ RACE clone. It is 87 nucleotides long and contains a polyadenylation signal site (AATAAA) and a polyadenylated tail (Fig. 4). The calculated molecular weight of

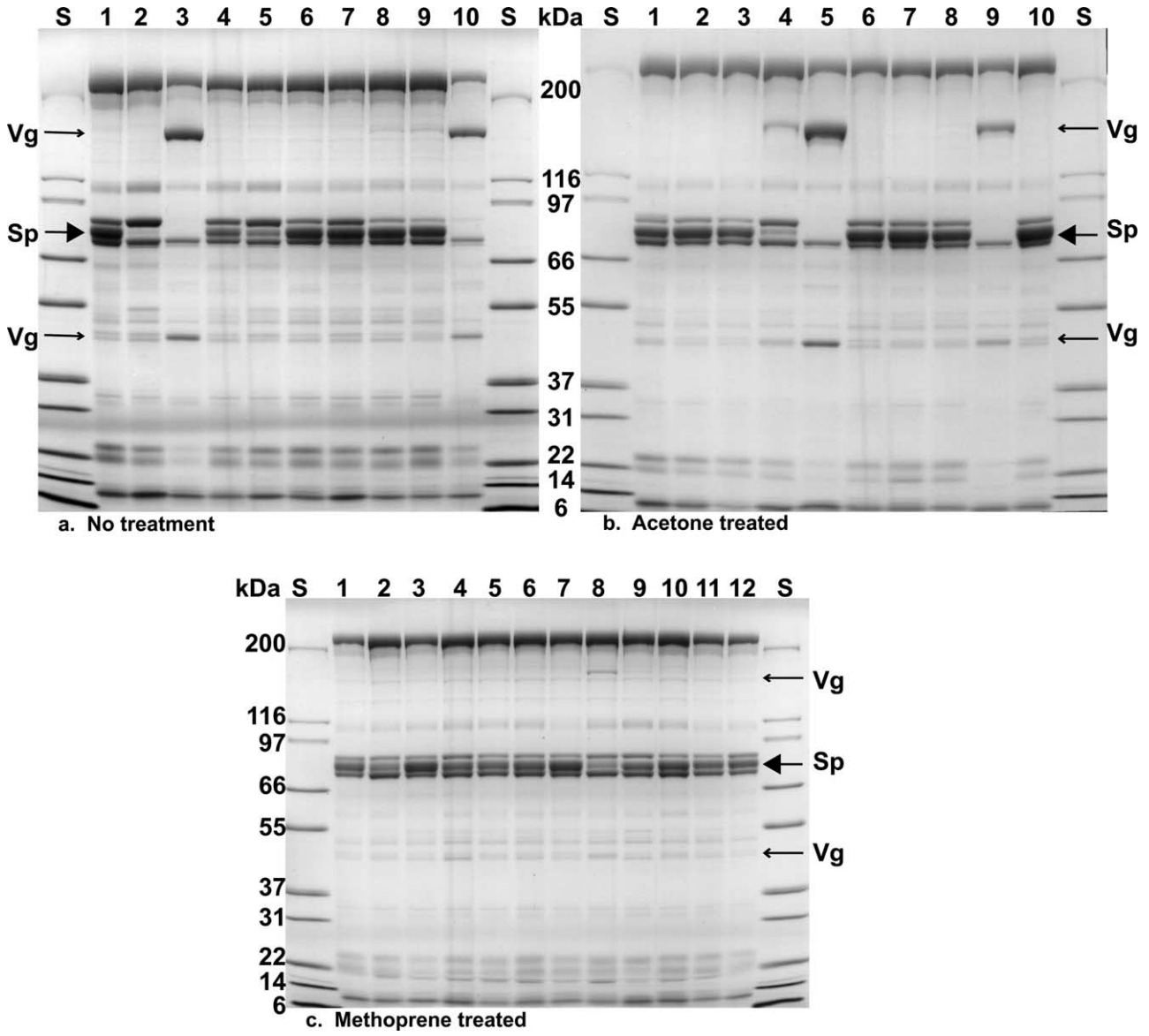

Fig. 3. Effect of methoprene on hemolymph polypeptides in female *A. grandis* held 53 days under diapause conditions. Hemolymph polypeptides were analyzed using SDS-PAGE (exponential gradient consisting of 6–16% acrylamide and 0.16–0.85% bis-acrylamide). Weevils were held for 14 days on the diapause feeding regime {29.4 °C, 11:13 (L:D), fed one boll (20–25 mm diameter) per 10 weevils three times weekly, water provided, ad lib}. When the median age was 14 days, weevils were transferred to a survival cage containing water only and maintained at 18.3 °C, 11:13 (L:D) for another 30 days. Weevils were: (a) untreated; (b) treated with 1 μl of acetone; or (c) treated with 1 μl (1 μg/μl) of methoprene zero, three, and six days following the starvation period. Hemolymph samples were collected nine days after the starvation period and the condition of the fat body and ovaries was assessed. Each lane was loaded with 8 μg of soluble polypeptides from the hemolymph of a single female. AgSP-1 (arrow), both subunits of VG (arrow), and the molecular weight standards (S) are indicated.

AgSP-1, including the 5′ untranslated region and the polyadenylated tail, is 2.78 kB. The nucleotide sequence has been submitted to the GenBank™/EMBL DataBank accession number AF512519.

3.5. Northern blot analysis

AgSP-1 and AgVG gene expression in the fat body of reproductive and diapausing weevils were analyzed to compare relative levels of expression under the two physiological states. Based on mRNA levels, AgVG expression was high in reproductive females, but was reduced significantly in diapausing females (Fig. 5). AgSP-1 mRNA was abundant in the fat bodies of both diapausing male and female weevils, whereas reproductive weevils of both sexes showed reduced levels of AgSP-1 expression. These results indicated an inverse correlation between AgVG and AgSP-1 gene expression in females. No apparent differences in AgSP-1 expression were observed between sexes. The estimated transcript size of AgSP-1 was 2.5 kB. The estimated molecular weight of AgVG (5.8 kB) was consistent with

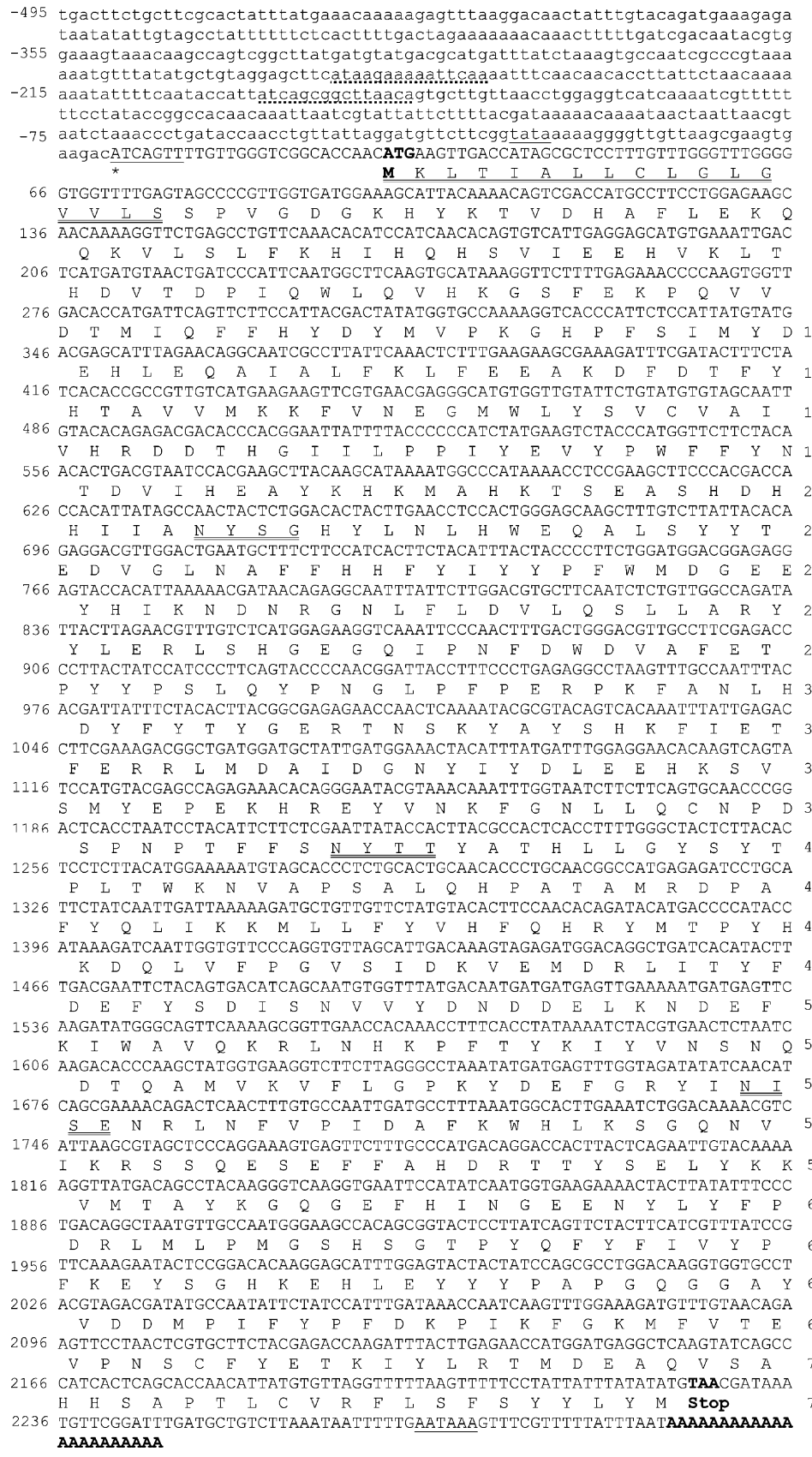

Fig. 4. Complete cDNA sequence including the 5′ untranslated region and the deduced amino acid sequence of AgSP-1 (accession no., AF512519). The complete sequence is composed of two separate fragments; the 2.2-kB genomic clone and the 1.0-kB 3′ RACE clone. Nucleotides are numbered from the transcription start site (∗=+1). Solid underlines indicate the putative TATA box, the transcription start site and the polyadenylation signal, respectively. The translation start ($^{+}$27), the stop codon ($^{+}$2270), and the poly-A tail ($^{+}$2294) are in bold. The double underlines indicate the putative signal peptide and three putative N-glycosylation sites. The dotted underlines indicate potential promoter regions with homology to the H-box region (−196; ATCAGCGGCTTAACA and −259; ATAAGAAAAATTCAA).

Table 1
Comparison of the deduced amino acid (% composition) of *A. grandis* with two other arylphorin-like hexamerins

Amino acid	AgSp-1	LdDP1	CvLSP-2
A Ala	2.75	3.05	2.54
C Cys	0.48	0.63	0.25
D Asp	5.81	5.64	7.95
E Glu	6.97	6.96	6.37
F Phe	**9.32**	**9.92**	**8.35**
G Gly	2.08	2.66	2.25
H His	7.08	4.04	5.92
I Ile	4.51	4.02	4.61
K Lys	7.07	6.28	6.80
L Leu	6.77	6.24	7.81
M Met	3.23	3.38	1.62
N Asn	4.28	5.73	5.34
P Pro	4.56	4.64	3.71
Q Gln	3.91	3.77	3.32
R Arg	3.66	7.27	4.62
S Ser	3.98	3.74	3.97
T Thr	3.91	2.97	2.74
V Val	5.00	4.49	5.86
W Trp	1.97	2.74	2.07
Y Tyr	**12.63**	**11.80**	**3.88**

The deduced mature protein of AgSP-1 (717 residues) was compared with hexamerins from *L. decemlineata* (LdDP1=diapause protein 1, X76080) and *Calliphora vicina* (CvLSP-2=larval serum protein LSP-2, U89789). The aromatic amino acids characteristic of arylphorins, Phe and Tyr, are in bold.

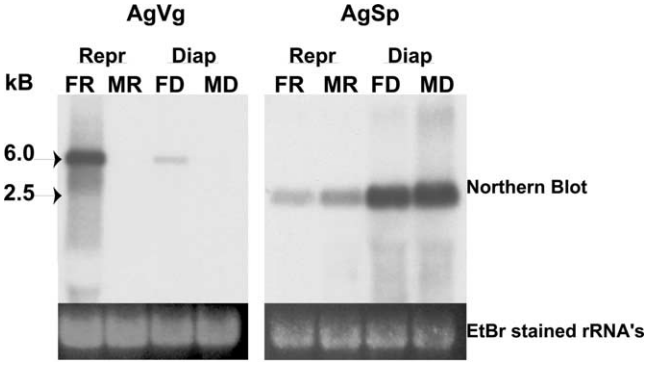

Fig. 5. Northern blot analysis of VG (6.0 kB) and AgSP-1 (2.3 kB) in the fat body of reproductive and diapausing *A. grandis*. Total RNA (10 μg for VG blot; 5 μg for AgSP-1 blot) from reproductive females (FR), reproductive males (MR), diapausing females (FD) and diapausing males (MD) was probed with a ^{32}P-labeled *A. grandis* specific VG probe (AgVG) or an *A. grandis* storage protein probe (AgSp). Equal transfer of each tissue type was confirmed by visualization of the ethidium bromide stained rRNA's following transfer. Hybridization was at 48 °C using UltraHyb (Ambion) for 12–14 h. Washes were performed at 65°C (2*5min—2 × SSC, 0.1% SDS; 2*15min—0.1 × SSC;0.1%SDS). Blots were exposed to film for approximately 1 h.

the molecular weight (6.0 kB) reported by Trewitt et al. (1992).

3.6. Multiple alignment and phylogenetic analysis

The deduced amino acid sequence of AgSP-1 was aligned with four proteins containing high aromatic amino acid contents from *Tenebrio molitor* (Tm), *L. decemlineata* (Ld), *Periplaneta americana* (Pa), and *Calliphora vicina* (Cv), and of two proteins from *Spodoptera litura* (Sl) and *Bombyx mori* (Bm)that are either moderately methionine-rich (Sl) or contain a high percentage of methionine (Bm) (Fig. 6). The alignment and percent identity was calculated based on the Clustal method (Higgins and Sharp, 1988). The deduced amino acid sequence of AgSP-1 showed the highest sequence identity with the two arylphorins from the coleopteran species *T. molitor* (43.2%) and *L. decemlineata* (38.3%), and with the other two arylphorin-like proteins from *P. americana* (30.4%) and *C. vicina* (30.2%).

Phylogenetic analysis (Fig. 7) grouped the arylphorin hexamerins in a single clade distinct from a clade containing a higher proportion of methionine. Thus, AgSP-1 appears to be derived from an arylphorin ancestor. AgSP-1 clusters with the other two coleopteran hexamerins, which appear to share a coleopteran ancestor not shared by the allergen of *P. americana* and the LSP-2 hexamerin of *C. vicina*. Although *C. vicina* LSP-2 contains a high percentage of Phe and Tyr (Table 1) suggesting that it is an arylphorin, Burmester et al. (1998a) reported strong phylogenetic evidence to support separation of LSP-2 from other arylphorin hexamerins, including both *C. vicina* calliphorin and *D. melanogaster* LSP-1 arylphorin. The parsimony tree supports this view more than the distance-based neighbor-joining tree, which is consistent with taxonomic affiliations where the arylphorin from the hemimetabolous cockroach is the most primitive and is from a line ancestral to the *C. vicina* hexamerin.

4. Discussion

Arylphorins are a class of storage hexamerins that contain a higher proportion (18–25%) of the aromatic amino acids, Phe and Tyr than most average proteins (King and Jukes, 1969; Kramer et al., 1980; Telfer et al., 1983). We identified a 77-kDa diapause-specific storage hexamerin in diapausing adults of the boll weevil, *A. grandis* (Fig. 1). Its size is consistent with other insect hexamerins and the deduced amino acid composition (Table 1) indicates that AgSP-1 is like an arylphorin, given its high concentration of aromatic amino acids (~21% by composition, Phe+Tyr) (Munn and Greville, 1969).

Diapausing weevils displayed high AgSP-1 titers in the hemolymph (Figs. 1 and 2) and high mRNA levels in the fat body (Fig. 5), while VG and VG mRNA were minimal in the hemolymph and fat body of diapausing

Fig. 6. Multiple alignment of the deduced amino acid sequence of AgSp-1 with other related hemolymph proteins. Abbreviations and accession numbers are as follows: Ag, *A. grandis* storage protein (AF512519); Tm, *T. molitor* hexamerin 2 precursor (AF395329); Ld, *L. decemlineata* diapause protein 1 (X76080); Pa, *P. americana* C12 allergen Cr-PI (L40818); Cv, *C. vicina* larval serum protein LSP-2 (U89789); Sl, *S. litera* moderately methionine-rich storage protein SL-2α (AJ249468); Bm, *B. mori* sex-specific storage protein SP1 (X12978).

females. The inverse was observed in reproductive females. VG was abundant in the hemolymph of reproductive females (Figs. 1 and 2) and highly expressed in the fat body (Fig. 5) and AgSP-1 was barely perceptible or absent. Hemolymph AgSP-1 and AgSP-1 mRNA were also minimal in non-diapause males. In many insects, the onset of VG expression/synthesis is stimulated by JH (Wyatt, 1988) thus, the inverse correlation between AgSP-1 and VG suggested that AgSP-1 might be suppressed by JH.

JH regulates stored hexamerins in several insect species. In *R. clavatus*, two of the four cyanoproteins (CP1–CP4) identified as storage hexamerins are differentially regulated by JH. In reproductive, non-diapause females,

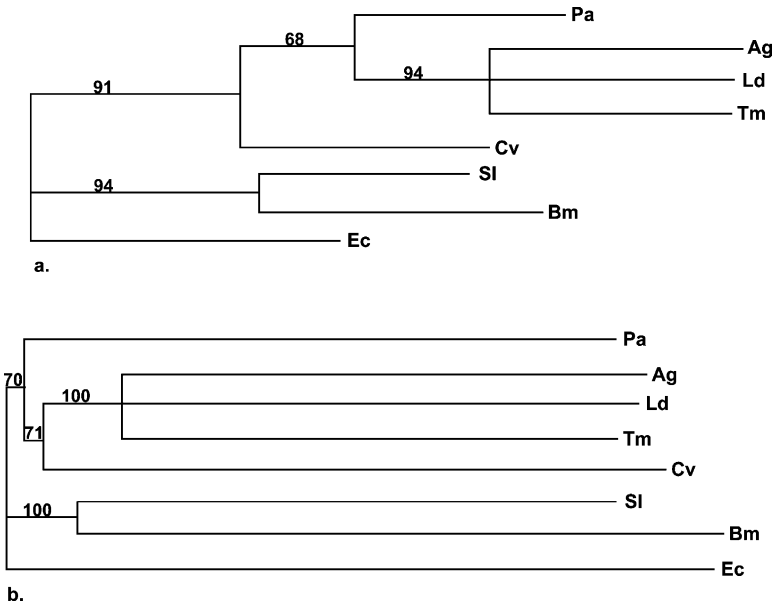

Fig. 7. Phylogenetic reconstructions for the storage protein from *A. grandis* and other related insect hexamerins. Maximum parsimony (a) and bootstrap (neighbor-joining method) (b) analyses were conducted using PAUP version 4.0 (Swofford, 1998). Percent Bootstrap support (1000 replicates) for each clade is indicated above the lines. Branch lengths are proportional to substitutions (a) or distance (b). Each tree was rooted using *Eurypelma californicum* hemocyanin subunit f, hcF (Ec; AJ277491), as the outgroup. Abbreviations and accession numbers are as follows: Pa, *P. americana* C12 allergen Cr-PI (L40818); Ag, *A. grandis* storage protein 1 AgSP-1 (AF512519); Ld, *L. decemlineata* diapause protein 1 (X76080); Tm, *T. molitor* hexamerin 2 precursor (AF395329); Cv, *C. vicina* larval serum protein LSP-2 (U89789); Sl, *S. litera* moderately methionine-rich storage protein SL-2α (AJ249468); Bm, *B. mori* sex-specific storage protein SP1 (X12978).

only CP1 is synthesized throughout the reproductive stages (Chinzei et al., 1992); in diapause-oriented females, CP4 is the predominant storage hexamerin. Methoprene treatment of diapause-oriented females effectively induces CP1 synthesis and inhibits CP4 synthesis (Chinzei et al., 1992; Miura et al., 1998).

In adult females of *Blaberus discoidalis* and in last instar larvae and short-day adults of *L. decemlineata*, gene expression of the arylphorins, *B*Ar and DP1 respectively, is suppressed by JH (De Kort and Koopmanschap, 1994; Jamroz et al., 1996). JH also suppresses synthesis and/or expression of several larval storage hexamerins in Lepidoptera (Corpuz et al., 1991; Hwang et al., 2001; Jones et al., 1993; Memmel et al., 1994).

Our data suggest that JH neither enhances nor suppresses AgSP-1 synthesis/expression in diapausing *A. grandis* weevils. No obvious differences were evident between AgSP-1 hemolymph titers from methoprene-treated diapausing weevils and the two controls (Fig. 3). Our work is in contrast to those for several other insects, in which JH has been shown to play an important role in hexamerin synthesis, however more research is necessary to fully define the influence of JH in boll weevil diapause.

Identification of AgSP-1 from the hemolymph required N-terminal analysis of the isolated polypeptide from SDS-PAGE. The estimated molecular weight of AgSP-1 (2.5 kB) based on Northern blot analysis (Fig. 5) was comparable to the calculated molecular weight (2.78 kB) of the gene sequence and to the reported size for other insect hexamerins (Burmester et al., 1998b; Jamroz et al., 1996; Koopmanschap et al., 1995; Palli et al., 1998; Sakurai et al., 1988a).

The genomic DNA library permitted us to identify possible regulatory features in the 5′ untranslated region. In addition to the TATA box located at −32, a potential H-box was observed at −196 (ATCAGCGGCTTAA CA) and −259 (ATAAGAAAAATTCAA) (Fig. 4) with 60 and 66.7% sequence identities, respectively, to the *Drosophila melanogaster* H-box present in the yolk polypeptide genes (Yan et al., 1987). A portion of the H-box sequence is homologous to vertebrate steroid hormone receptor binding sites and to the ecdysone control region of the *D. melanogaster hsp23* gene (Yan et al., 1987).

Further analysis of these two regions in the 5′ untranslated region of AgSP-1 (−196 and −259), however, showed little similarity to the ecdysone response element of the *D. melanogaster hsp27* gene (Riddihough and Pelham, 1987) or to the vertebrate glucocorticoid (GRE) and estrogen receptor (ERE) binding sites (Beato, 1989). A critical feature necessary for binding the *D. melanogaster hsp27* gene to the ecdysteroid receptor is an imperfect palindromic sequence (GGTTCAATGCACT) (Ożyhar et al., 1991). The GRE and ERE binding sites are also characterized by palindromic sequences (GGTACAnnnTGTYCT and NGGTCAnnnTGACCN, respectively) (Beato, 1989; Ożyhar et al., 1991). Such a

sequence was not observed in the 5′ untranslated region of AgSP-1.

Many eukaryotic genes contain a regulatory region called a CAAT box that promotes efficient transcription initiation (Benoist et al., 1980). This region does not appear to be present in AgSP-1. Storage hexamerins from *B. mori* and *Sarcophaga peregrina* also lack the CAAT box (Fujii et al., 1989; Matsumoto et al., 1986).

The first 17 amino acids of the deduced AgSp-1 protein, including the ATG translation start codon, consisted of many hydrophobic amino acids, typical for a signal peptide. The size of the putative AgSP-1 signal peptide is consistent with those of other insect storage hexamerins, which vary from 15 to 18 amino acids (Burmester et al., 1998b; Cho et al., 1999; Fujii et al., 1989; Jamroz et al., 1996; Jones et al., 1990; Memmel et al., 1992; Memmel et al., 1994; Sakurai et al., 1988b; Willott et al., 1989). The calculated molecular weight of the AgSP-1 deduced amino acid sequence, less the signal peptide, was 85.3 kDa. This weight differed from the 77-kDa size estimated by SDS-PAGE. The storage hexamerins from *Galleria mellonella*, *Aedes aegypti*, and *D. melanogaster* show similar size discrepancies (Gordadze et al., 1999; Massey et al., 1997; Memmel et al. (1992).

Comparison of the AgSP-1 deduced amino acid sequence (accession no. AF512519) with the sequences in GenBank showed the highest similarity to two genes from *T. molitor*, an arylphorin (unpublished, accession no. AF395329) and an 86-kDa early encapsulation larval storage protein (Cho et al., 1999). AgSP-1 was also most similar to diapause protein 1 from *L. decemlineata* (Koopmanschap et al., 1995), two aromatic amino acid-rich allergens from *P. americana* (Wu et al., 1996) and the arylphorin hexamerin from *B. discoidalis* (Jamroz et al., 1996). AgSP-1 was less similar to the larval serum protein LSP-2 from *C. vicina* (Palli et al., 1998), the methionine-rich proteins of *S. litura* and the sex-specific methionine-rich storage protein, SP1 from *B. mori* (Sakurai et al., 1988a). The aromatic amino acid content (Table 1) as well as the high sequence (Fig. 6) and phylogenetic similarity (Fig. 7) with coleopteran hexamerins, clearly indicate that AgSP-1 is an arylphorin.

Storage hexamerins have diverse functions and are widely present in other stages of development. In the adults of several ant species, storage hexamerins serve as an amino acid reserve for queens during their single period of brood rearing (Lewis et al., 2001; Martinez and Wheeler, 1994; Wheeler and Buck, 1995). The storage hexamerin (CP1) from the bean bug, *R clavatus*, is present in both the nymphal and adult stages and is a major protein constituent of the egg yolk (Chinzei et al., 1992). Storage hexamerins also function as binding proteins for JH and riboflavin (Braun and Wyatt, 1996; Miller and Silhacek, 1995; Shapiro et al., 1992). In *A. grandis*, Ag-SP1 is likely an important amino acid reserve during diapause.

Our research has identified a reliable biochemical indicator of diapause in *A. grandis*. Using standardized conditions (Spurgeon and Raulston, 1998b; Spurgeon and Esquivel, 2000), AgSP-1 was consistently high in adults of both sexes exhibiting diapause characters and was reduced or absent in reproductive weevils. Analysis of AgSP-1 levels in males and females, in addition to VG abundance in females, has the potential to be an effective tool for evaluating diapause status.

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. Journal of Molecular Biology 215, 403–410.

Beato, M., 1989. Gene regulation by steroid hormones. Cell 56, 335–344.

Benoist, C., O'Hare, K., Breathnach, R., Chambon, P., 1980. The ovalbumin gene-sequence of putative control regions. Nucleic Acids Research 8, 127–142.

Betz, N.L., Lambremont, E.N., 1967. Changes in stored glycogen and lipid associated with diapause of the adult boll weevil, *Anthonomus grandis* (Coleoptera: Curculionidae). Annals of the Entomological Society of America 60, 866–868.

Braun, R.P., Wyatt, G.R., 1996. Sequence of the hexameric juvenile hormone-binding protein from the hemolymph of *Locusta migratoria*. Journal of Biological Chemistry 271, 31756–31762.

Brazzel, J.R., Newsom, L.D., 1959. Diapause in *Anthonomus grandis* Boh. Journal of Economic Entomology 52, 603–611.

Breathnach, R., Chambon, P., 1981. Organization and expression of eucaryotic split genes coding for proteins. Annual Review of Biochemistry 50, 349–383.

Burmester, T., Massey, H.C. Jr., Zakharkin, S.O., Beněs, H., 1998a. The evolution of hexamerins and the phylogeny of insects. Journal of Molecular Evolution 47, 93–108.

Burmester, T., Kolling, C., Schroer, B., Scheller, K., 1998b. Complete sequence, expression, and evolution of the hexamerin LSP-2 of *Calliphora vicina*. Insect Biochemistry and Molecular Biology 28, 11–22.

Chinzei, Y., Miura, K., Kobayashi, L., Shinoda, T., Numata, H., 1992. Cyanoprotein: developmental stage, sex and diapause-dependent expression, and synthesis regulation by juvenile hormone in the bean bug, *Riptortus clavatus*. Archives of Insect Biochemistry and Physiology 20, 61–73.

Cho, M.Y., Choi, H.W., Moon, G.Y., Kim, M.H., Kwon, T.H.,

Homma, K., Natori, S., Lee, B.L., 1999. An 86 kDa diapause protein 1-like protein is a component of early-staged encapsulation-relating proteins in coleopteran insect, *Tenebrio molitor* larvae. FEBS Letters 451, 303–307.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium-thiocyanate-phenol-chloroform extraction. Analytical Biochemistry 162, 156–159.

Cobb, P.P., Bass, M.H., 1968. Some effects of photoperiod, temperature, and food on the induction of diapause in the boll weevil. Journal of Economic Entomology 61, 624–625.

Corpuz, L.M., Choi, H., Muthukrishnan, S., Kramer, K.J., 1991. Sequences of two cDNAs and expression of the genes encoding methionine-rich storage proteins of *Manduca sexta*. Insect Biochemistry 21, 265–276.

De Kort, C.A.D., Koopmanschap, A.B., 1994. Nucleotide and deduced amino acid sequence of a cDNA clone encoding diapause protein 1, an arylphorin-type storage hexamer of the Colorado potato beetle. Journal of Insect Physiology 40, 527–535.

Earle, N.W., Newsom, L.D., 1964. Initiation of diapause in the boll weevil. Journal of Insect Physiology 10, 131–139.

Fujii, T., Sakurai, H., Izumi, S., Tomino, S., 1989. Structure of the gene for the arylphorin-type storage protein SP 2 of *Bombyx mori*. Journal of Biological Chemistry 264, 11020–11025.

Gannon, F., O'Hare, K., Perrin, F., LePennec, J.P., Benoist, C., Cochet, M., Breathnach, R., Royal, A., Garapin, A., Cami, B., Chambon, P., 1979. Organisation and sequences at the 5′ end of a cloned complete ovalbumin gene. Nature 278, 428–435.

Gordadze, A.V., Korochkina, S.E., Zakharkin, S.O., Norton, A.L., Benes, H., 1999. Molecular cloning and expression of two hexamerin cDNAs from the mosquito, *Aedes aegypti*. Insect Molecular Biology 8, 55–66.

Guerra, A.A., Garcia, R.D., Tamayo, J.A., 1982. Physiological activity of the boll weevil during the fall and winter in subtropical areas of the Rio Grande valley of Texas. Journal of Economic Entomology 75, 11–15.

Guerra, A.A., Wiygul, G., Garcia, R.D., 1983. Oxygen consumption in boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), from subtropical areas of the Rio Grande valley of Texas. Comparative Biochemistry and Physiology 74A, 263–265.

Haunerland, N.H., 1996. Insect storage proteins—gene families and receptors. Insect Biochemistry and Molecular Biology 26, 755–765.

Hagedorn, H.H., Kunkel, J.G., 1979. Vitellogenin and vitellin in insects. Annual Review of Entomology 24, 475–505.

Higgins, D.G., Sharp, P.M., 1988. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene 73, 237–244.

Hultmark, D., Klemenz, R., Gehring, W.J., 1986. Translational and transcriptional control elements in the untranslated leader of the heat-shock gene *hsp22*. Cell 44, 429–438.

Hwang, S.-J, Cheon, H.-M., Kim, H.-J., Chae, K.-S., Chung, D.-H., Kim, M.-O., Park, J.-S., Seo, S.-J., 2001. cDNA sequence and gene expression of storage protein-2—a juvenile hormone-suppressible hexamerin from the fall webworm, *Hyphantria cunea* Drury. Comparative Biochemistry and Physiology 129 (Part B), 97–107.

Jamroz, R.C., Beintema, J.J., Stam, W.T., Bradfield, J.Y., 1996. Aromatic hexamerin subunit from adult female cockroaches (*Blaberus discoidalis*): molecular cloning, suppression by juvenile hormone, and evolutionary perspectives. Journal of Insect Physiology 42, 115–124.

Jones, G., Manczak, M., Horn, M., 1993. Hormonal regulation and properties of a new group of basic hemolymph proteins expressed during insect metamorphosis. Journal of Biological Chemistry 268, 1284–1291.

Jones, G., Brown, N., Manczak, M., Hiremath, S., Kafatos, F.C., 1990. Molecular cloning, regulation, and complete sequence of a hemocyanin-related, juvenile hormone-suppressible protein from insect hemolymph. Journal of Biological Chemistry 265, 8596–8602.

Kanost, M.R., Kawooya, J.K., Law, J.H., Ryan, R.O., Van Heusden, M.C., Ziegler, R., 1990. Insect haemolymph proteins. Advances in Insect Physiology 22, 299–396.

Keeley, L.L., Moody, D.S., Lynn, D., Joiner, R.L., Vinson, S.B., 1977. Succinate-cytochrome *c* reductase activity and lipids in diapause and non-diapause *Anthonomus grandis* from different latitudes. Journal of Insect Physiology 23, 231–234.

King, J.L., Jukes, T.H., 1969. Non-Darwinian evolution. Science 164, 788–798.

Koopmanschap, A.B., Lammers, J.H.M., De Kort, C.A.D., 1995. The structure of the gene encoding diapause protein 1 of the Colorado potato beetle (*Leptinotarsa decemlineata*). Journal of Insect Physiology 41, 509–518.

Kramer, S.J., Mundall, E.C., Law, J.H., 1980. Purification and properties of manducin, an amino acid storage protein of the haemolymph of larval and pupal *Manduca sexta*. Insect Biochemistry 10, 279–288.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lambremont, E.N., 1961. Homogenate respiration of diapausing and nondiapausing boll weevils. Annals of the Entomological Society of America 54, 313–316.

Lambremont, E.N., Blum, M.S., Schrader, R.M., 1964. Storage and fatty acid composition of triglycerides during adult diapause of the boll weevil. Annals of the Entomological Society of America 57, 526–532.

Lefevere, K.S., Koopmanschap, A.B., De Kort, C.A.D., 1989. Changes in the concentrations of metabolites in haemolymph during and after diapause in female Colorado potato beetle, *Leptinotarsa decemlineata*. Journal of Insect Physiology 35, 121–128.

Lewis, D.K., Campbell, J.Q., Sowa, S.M., Chen, M.-E., Vinson, S.B., Keeley, L.L., 2001. Characterization of vitellogenin in the red imported fire ant, *Solenopsis invicta* (Hymenoptera: Apocrita: Formicidae). Journal of Insect Physiology 47, 543–552.

Lloyd, E.P., Tingle, F.C., Gast, R.T., 1967. Environmental stimuli inducing diapause in the boll weevil. Journal of Economic Entomology 60, 99–102.

Martinez, T., Wheeler, D., 1991. Identification of vitellogenin in the ant, *Camponotus festinatus*: changes in hemolymph proteins and fat body development in workers. Archives of Insect Biochemistry and Physiology 17, 143–155.

Martinez, T., Wheeler, D.E., 1994. Storage proteins in adult ants (*Camponotus festinatus*): roles in colony founding by queens and in larval rearing by workers. Journal of Insect Physiology 40, 723–729.

Massey, H.C. Jr., Kejzlarová-Lepesant, J., Willis, R.L., Castleberry, A.B., Beněs, H., 1997. The *Drosophila Lsp-1b* gene a stuctural and phylogenetic analysis. European Journal of Biochemistry 245, 199–207.

Matsumoto, N., Nakanishi, Y., Natori, S., 1986. Homologies of nucleotide sequences in the 5′-end regions of two developmentally regulated genes of *Sarcophaga peregrina*. Nucleic Acids Research 14, 2685–2698.

Memmel, N.A., Trewitt, P.M., Silhacek, D.L., Kumaran, A.K., 1992. Nucleotide sequence and structure of the arylphorin gene from *Galleria mellonella*. Insect Biochemistry and Molecular Biology 22, 333–342.

Memmel, N.A., Trewitt, P.M., Grzelak, K., Rajaratnam, V.S., Kumaran, A.K., 1994. Nucleotide sequence, structure and developmental regulation of LHP82, a juvenile hormone-suppressible hexamerin gene from the waxmoth, *Galleria mellonella*. Insect Biochemistry and Molecular Biology 24, 133–144.

Miller, S.G., Silhacek, D.L., 1995. Riboflavin binding proteins and flavin assimilation in insects. Comparative Biochemistry and Physiology 110B, 467–475.

Miura, K., Shinoda, T., Yura, M., Nomura, S., Kamiya, K., Yuda, M., Chinzei, Y., 1998. Two hexameric cyanoprotein subunits from an

insect, *Riptortus clavatus* sequence, phylogeny and developmental and juvenile hormone regulation. European Journal of Biochemistry 258, 929–940.

Munn, E.A., Greville, G.D., 1969. The soluble proteins of developing *Calliphora erythrocephala*, particularly calliphorin, and similar proteins in other insects. Journal of Insect Physiology 15, 1935–1950.

Nettles, W.C. Jr., Betz, N.L., 1965. Glycogen in the boll weevil with respect to diapause, age, and diet. Annals of the Entomological Society of America 58, 721–726.

Nettles, W.C. Jr., Parro, B., Sharbaugh, C., Mangum, C.L., 1972. Trehalose and other carbohydrates in diapausing and starving boll weevils. Annals of the Entomological Society of America 65, 554–558.

Ożyhar, A., Strangmann-Diekmann, M., Kiltz, H.-H., Pongs, O., 1991. Characterization of a specific ecdysteroid receptor-DNA complex reveals common properties for invertebrate and vertebrate hormone-receptor/DNA interactions. European Journal of Biochemistry 200, 329–335.

Palli, S.R., Ladd, T.R., Ricci, A.R., Primavera, M., Mungrue, I.N., Pang, A.S.D., Retnakaran, A., 1998. Synthesis of the same two proteins prior to larval diapause and pupation in the spruce budworm, *Choristoneura fumiferana*. Journal of Insect Physiology 44, 509–524.

Puissant, C., Houdebine, L.-M., 1990. An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. BioTechniques 8, 148–149.

Raikhel, A.S., Dhadialla, T.S., 1992. Accumulation of yolk proteins in insect oocytes. Annual Review of Entomology 37, 217–251.

Riddihough, G., Pelham, H.R.B., 1987. An ecdysone response element in the *Drosophila hsp27* promoter. EMBO Journal 6, 3729–3734.

Sakurai, H., Fujii, T., Izumi, S., Tomino, S., 1988a. Complete nucleotide sequence of gene for sex-specific storage protein of *Bombyx mori*. Nucleic Acids Research 16, 7717–7719.

Sakurai, H., Fujii, T., Izumi, S., Tomino, S., 1988b. Structure and expression of gene coding for sex-specific storage protein of *Bombyx mori*. Journal of Biological Chemistry 263, 7876–7880.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sappington, T.W., Spurgeon, D.W., 2000. Preferred technique for adult sex determination of the boll weevil (Coleoptera: Curculionidae). Annals of the Entomological Society of America 93, 610–615.

Shapiro, J.P., Silhacek, D.L., Niedz, R.P., 1992. Storage proteins of the larval root weevil *Diaprepes abbreviatus* (Coleoptera: Curculionidae): riboflavin binding and subunit isolation. Archives of Insect Biochemistry and Physiology 20, 315–331.

Sowa, B.A., Kelly, K.A., Ficht, T.A., Frey, M., Adams, L.G., 1991. SDS-soluble and peptidoglycan-bound proteins in the outer membrane-peptidoglycan complex of *Brucella abortus*. Veterinary Microbiology 27, 351–369.

Spurgeon, D.W., Raulston, J.R., 1996. Boll weevil reproductive development responses to crowding and variations in host quality. In: Proceedings of the Beltwide Cotton Conference. National Cotton Council, Memphis, TN, pp. 983–987.

Spurgeon, D.W., Raulston, J.R., 1998a. Diapause induction in subtropical boll weevils. In: Proceedings of the Beltwide Cotton Conference. National Cotton Council, Memphis, TN, pp. 1019–1025.

Spurgeon, D.W., Raulston, J.R., 1998b. Boll weevil (Coleoptera: Curculionidae) reproductive development as a function of temperature. Environmental Entomology 27, 675–681.

Spurgeon, D.W., Esquivel, J.F., 2000. Effect of food type and feeding rate on boll weevil diapause. In: Proceedings of the Beltwide Cotton Conference. National Cotton Council, Memphis, pp. 1073–1076.

Sterling, W., 1972. Photoperiodic sensitivity in the ontogeny of the boll weevil. Environmental Entomology 1, 568–571.

Swofford, D.L., 1998. Phylogenetic Analysis Using Parsimony, Version 4.0. Sinauer Associates, Sunderland, MA.

Taub-Montemayor, T.E., Rankin, M.A., 1997. Regulation of vitellogenin synthesis and uptake in the boll weevil, *Anthonomus grandis*. Physiological Entomology 22, 261–268.

Taub-Montemayor, T.E., Dahm, K.H., Bhaskaran, G., Rankin, M.A., 1997a. Rates of juvenile hormone biosynthesis and degradation during reproductive development and diapause in the boll weevil, *Anthonomus grandis*. Physiological Entomology 22, 269–276.

Taub-Montemayor, T.E., Palmer, J.O., Rankin, M.A., 1997b. Endocrine regulation of reproduction and diapause in the boll weevil, *Anthonomus grandis* Boheman. Archives of Insect Biochemistry and Physiology 35, 455–477.

Telfer, W.H., Kunkel, J.G., 1991. The function and evolution of insect storage hexamers. Annual Review of Entomology 36, 205–228.

Telfer, W.H., Keim, P.S., Law, J.H., 1983. Arylphorin, a new protein from *Hyalophora cecropia*: comparisons with calliphorin and manducin. Insect Biochemistry 13, 601–613.

Trewitt, P.M., Heilmann, L.J., Degrugillier, S.S., Kumaran, A.K., 1992. The boll weevil vitellogenin gene: nucleotide sequence, structure, and evolutionary relationship to nematode and vertebrate vitellogenin genes. Journal of Molecular Evolution 34, 478–492.

Wagner, T.L., Villavaso, E.J., 1999. Diapause in the boll weevil (Coleoptera: Curculionidae): seasonal occurrence in Mississippi populations. Annals of the Entomological Society of America 92, 382–395.

Wagner, T.L., Villavaso, E.J., Willers, J.L., 1999. Diapause in the boll weevil (Coleoptera: Curculionidae): life-stage sensitivity to environmental cues. Annals of the Entomological Society of America 92, 396–402.

Wheeler, D.E., Buck, N.A., 1995. Storage proteins in ants during colony development and colony founding. Journal of Insect Physiology 41, 885–894.

Willott, E., Wang, X.-Y., Wells, M.A., 1989. cDNA and gene sequence of *Manduca sexta* arylphorin, an aromatic amino acid-rich larval serum protein. Journal of Biological Chemistry 264, 19052–19059.

Wu, C.H., Lee, M.F., Liao, S.C., Luo, S.F., 1996. Sequencing analysis of cDNA clones encoding the American cockroach Cr-PI allergens. Journal of Biological Chemistry 271, 17937–17943.

Wyatt, G.R., 1988. Vitellogenin synthesis and the analysis of juvenile hormone action in locust fat body. Canadian Journal of Zoology 66, 2600–2610.

Yan, Y.L., Kunert, C.J., Postlethwait, J.H., 1987. Sequence homologies among the three yolk polypeptide (*Yp*) genes in *Drosophila melanogaster*. Nucleic Acids Research 15, 67–85.